Let op! Cell wall under construction

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INTRODUCTORY NOTE

Control experiments can trigger new stories

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The domain Bacteria contains single celled organisms that present a diverse spectrum of shapes. The structure responsible for these shapes is the cell wall. The cell wall provides a physical protection against the environment and it also allows bacteria to resist osmotic changes. The main component of the cell wall is peptidoglycan (PG), a polymer built up from glycan strands that are crosslinked via short peptide side chains[1]. The glycan strands consist of subunits formed by two sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), whereas the peptide side chains (stem peptides) have a species specific composition, are generally five amino-acids long, but may contain additional amino-acids that are branched off, and are partially composed of D-amino acids[1]. The amount of material, as well as the number of crosslinks between the glycan strands, are what determines the shape and strength of the cell wall.

Peptidoglycan synthesis is a tightly regulated process in which several enzymes are involved. This process begins with the synthesis of PG subunits, attached to a membrane embedded lipid carrier molecule, in the cytoplasm. This building block, known as Lipid II, is then flipped over the membrane and incorporated into a PG strand by a group of enzymes known as penicillin binding proteins (PBPs)[1](**Chapter 1**).

The proper localization of PBPs is important as it determines where PG synthesis will take place - cells in which PBPs do not localize correctly often have shape defects. Two main theories of how PBPs localize have been proposed. The first theory suggests that the interaction with cytoskeleton proteins like FtsZ and MreB controls the localization of the cell wall machinery[1]. In contrast, the second theory proposes that the presence of PG precursor is the factor that determines the proper position of the PBPs[2]. This second theory is also known as the 'substrate localization' theory and was originally proposed for organisms that lack MreB-like cytoskeletal proteins[2]. Studies on *Bacillus subtilis* and *Escherichia coli* demonstrated that when cell wall synthesis is depleted, MreB motility is decreased[3–5] supporting the idea that Lipid II is the key factor that determine the localization of the cell wall machineries and not the cytoskeleton proteins.

In Lages *et al*. 2013, we took a different approach to explore the substrate availability theory. Instead of depleting Lipid II, the precursor was delocalized from its usual position using nisin. Then, the position of the PBPs would follow by fluorescent microscopy. However, nisin treatment has as secondary effect the collapse of the membrane potential which affects the localization membrane proteins[6]. Therefore, only two *Bacillus* PBPs important during cell elongation, PBP2a and PbpH, and which are not affected by membrane potential were tested. After nisin treatment, Lipid II was

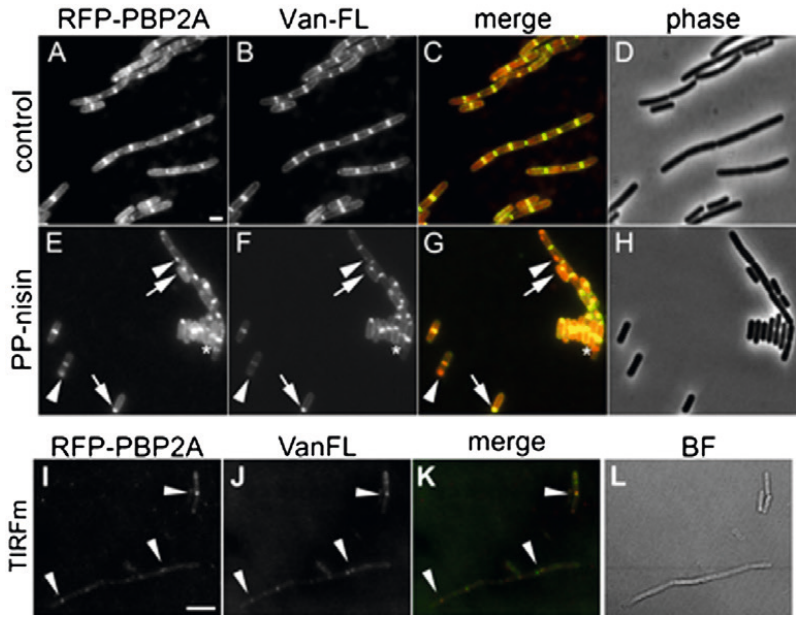

Figure 1. Colocalization of Lipid II and PBP2A in *B. subtilis*. Taken from [5]
A–H. Epi-fluorescence microscopy of *B. subtilis* strain 4042 imaged for RFP-PBP2A (A,E, red in overlay) and Van-FL (B,F, green in overlay), an overlay of the images (C,G) and a phase-contrast image of the cells (D,H) in untreated cells (A–D) or after treatment with PP-nisin (E–H). Scale bar same for all: 2 µm. Arrows indicate spots of clear colocalization, arrowheads indicate spots of colocalization with a weaker signal for Van-FL, and the asterisk indicates PBP2A fluorescence without Van-FL staining. In total, 496 cells were analysed, out of which 223 had Van-FL patches and 228 had PBP2A patches. Two hundred one cells contained patches of both Van-FL and PBP2A, and in 188 cells, these patches overlapped (94%). **I–L**. *Bacillus subtilis* strain 4042 labelled with Van-FL was imaged by TIRF microscopy after PP-nisin treatment for RFP-PBP2A (I) and Van-FL (J). (K) Merged signals for RFP-PBP2A and Van-FL; (L) bright field image of the cells. Arrow heads indicate the delocalized spots. Scale bar: 2 µm.

localized in spots that were overlapping with the fluorescent version of PBP2A (Figure 1) and PbpH. This result supports the hypothesis that Lipid II availability is responsible for the position of PBP2A and PbpH and not MreB[7].

During the development of Lages *et al*. 2013 a series of control experiments was performed. From those experiments two different observations led to the development of chapters 2 and 3 in this thesis.

1. SEARCHING FOR A TOOL

As mentioned previously, the strategy to test the substrate availability theory was to change the original position of the PG precursor and then check the

effects on the localization of the components of the PG synthesis machinery. To modify Lipid II localization we took advantage of one of the mechanisms of action of nisin. Nisin is a lantibiotic synthesized by *Lactococcus lactis*. It is composed of five lanthionine rings (A-E) connected by a flexible hinge between the 3rd and 4th ring[8] (**Chapter 2**, Figure 1). Nisin binds to the pyrophosphate cage of Lipid II via its two amino-terminal rings (A and B)[9], which blocks the addition of the Lipid II to PG via a glycosyl transferase reaction, and as a consequence the PG synthesis pathway is blocked (occlusion). After binding, the nisin C-terminal ring penetrates the membrane to form a pore that is composed of a stable complex of four Lipid II molecules and eight nisin molecules[10]. As a consequence of pore formation, the membrane potential collapses, the cell loses nutrients, and the cell will die. In addition to pore formation and occlusion, nisin has an extra mode of action. Nisin sequesters Lipid II by removing it from its original localization and to form clusters[11]. It is this last mechanism of action that was used as a tool to study the substrate availability theory.

It has been reported that some membranes proteins delocalize when there is a change in the membrane potential[3]. Therefore, using nisin has a major issue as it forms pores that collapse the membrane potential. Then, distinguishing between the change of localization of PBPs as an effect of membrane potential or as Lipid II clustering would not be possible. To try to avoid this issue, the experiments were performed with a nisin variant that has been reported to not form pores: PP-nisin [N20P/M21P].

PP-nisin has a replacement of two amino acids in the hinge region, asparagine(20) and methionine (21) to two prolines. PP-nisin keeps its antimicrobial activity, but it has lost the ability to form pores *in vitro*[4,5]. As part of the control experiments, the effect of PP-nisin on the membrane potential of *Bacillus* was tested. For this purpose the fluorescent membrane sensitive dye $DiSC_3(5)$ was used. Disc3(5) accumulates in polarized membranes where the fluorescence is quenched. However, when the membrane potential is dissipated the dye is released and the fluorescence increases. Surprisingly, PP-nisin was able to depolarize *Bacillus* membranes [7] (Figure 2).

As the membrane potential dissipation produced by PP-nisin was an obstacle to study the substrate availability theory, we tested other nisin variants with the aim to find a nisin mutant which could delocalize Lipid II without affecting the membrane potential. We did not find any such variant, but this search let us get into the mechanism of action of nisin which resulted in **chapter 2** of this thesis. In the end, we used PP-nisin to delocalize Lipid II, but restricted our study to the effects of Lipid II delocalization to proteins for which the localization is not changed upon membrane potential dissipation.

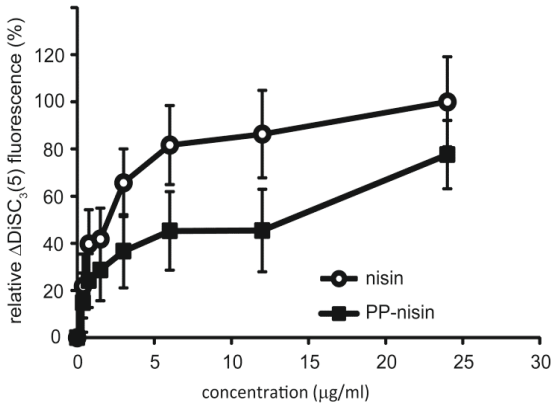

Figure 2. Fluorimetric measurement of the collapse of membrane potential by nisin and PP-nisin. Taken from [7].
The ΔΨ-sensitive fluorescent dye $DiSC_3(5)$ accumulates on polarized membranes of glucose energized *B. subitilis* cells, which results in fluorescence quenching. Dissipation of ΔΨ by nisin or PP-nisin is measured as release of the dye to the medium resulting in an increase in fluorescence. Various concentrations of nisin and PP-nisin were tested; values represent the mean and standard deviation from three different experiments that were performed in duplicate. Addition of nisin or PP-nisin at concentrations used to delocalize Lipid II (1.5 μg/ml) resulted in a significant increase in $DiSC_3(5)$ fluorescence, indicative of a (partial) collapse of the membrane potential.

2. UNEXPECTED FLUORESCENCE AT THE DIVISION SITE

The story presented in **chapter 3** is also a result from an observation made during the development of Lages *et al*. 2013. It has been suggested that *Bacillus* division site is enriched with the precursor of the cell wall, Lipid II, as it is necessary to synthesize the new septum. This conclusion was made with the introduction of a fluorescent analogue of vancomycin (Van-FL)[11] which allows the detection of Lipid II by fluorescent microscopy. Vancomycin is an antibiotic that binds to the terminal D-Ala-D-Ala residues of the pentapeptide of the PG building block. Therefore, Van-FL allows to visualise Lipid II and building blocks which have been incorporated into the cell wall, but where the D-Ala-D-Ala has not been lost. A normal profile of *Bacillus* stained with Van-FL will show fluorescence at the division site and lateral wall, while fluorescence is absent from the poles. The fluorescence at the division site is present as a thick band, which lead to the conclusion that the concentration of Lipid II at the division site is high.

The aim in Lages *et al*. 2013 was to demonstrate that delocalized Lipid II is responsible for the delocalization of the components of the cell wall machinery. As part of the controls, Lipid II was depleted using fosfomycin

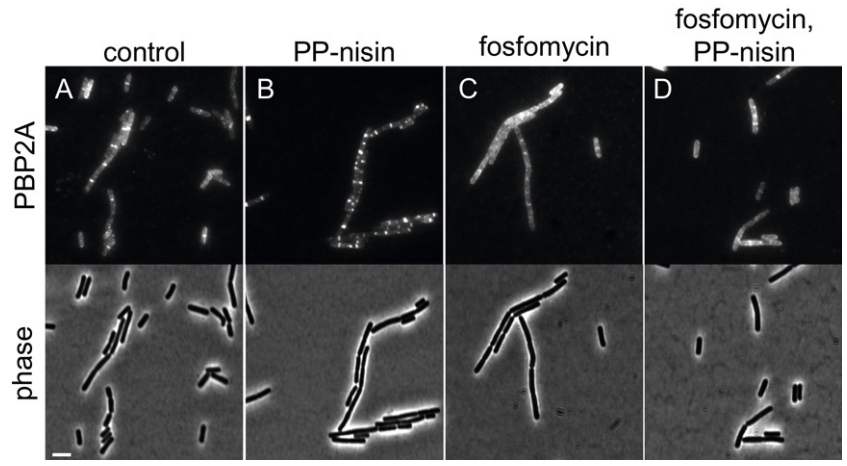

Figure 3. Lipid II is required for PBP2A patch formation. Taken from [7]
B. subtilis strain 4042 imaged for RFP-PBP2A (top) and phase-contrast image of the cells (bottom). Control cells were either not treated (A) or treated with PP-nisin only (B). Fosfomycin was used to block Lipid II production, and cells were not treated (C) or treated with PP-nisin (D). Patch formation only occurred in cells treated with PP-nisin in which Lipid II production was not blocked. Scale bar, same for A–D: 4 μm.

(Figure 3). Fosfomycin is an antibiotic that inhibits MurA, the first enzyme involved in the Lipid II synthesis pathway[15]. After Lipid II depletion, *Bacillus* was labelled with Van-FL in order to corroborate that Lipid II levels had been decreased. No Van-FL signal was detected at the lateral wall (data no shown), which indicated that the antibiotic treatment was effective. Intriguingly, Van-FL fluorescent signal was clearly visible at the division site. In order to try to reduce the signal at the septum, the incubation time with fosfosmycin and its concentration were increased. However, neither of these changes blocked the presence of Van-FL at the division site. This phenomenon caught our attention for two reasons. First, increasing the fosfomycin concentration and time of incubation effectively killed *Bacillus* which strongly suggested that Lipid II synthesis was inhibited. Second, Lipid II has a high turnover rate[8], which make it unlikely to stay at the division site for long time. This observation lead us to consider the possibility that Van-FL was not labeling Lipid II, but pre-existing unprocessed PG. Experiments that explore this hypothesis are presented on **Chapter 3**.

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